PROFESSOR DIOGO TEIXEIRA CARVALHO (Orcid ID : 0000-0002-6362-2822)

Article type : Research Article

Title: In vitro and in vivo trypanocidal activities of 8-methoxy-3-(4-nitrobenzoyl)-6-propyl-2*H*-cromen-2-one, a new synthetic coumarin of low cytotoxicity against mammalian cells

Short running title: New synthetic coumarin with trypanocidal activity

Key words: Coumarins, Nitro compounds, Trypanocidal agents, *Trypanosoma cruzi,* Chagas' disease.

Guilherme Andrade Brancaglion[a], André Eidi Toyota[a], José Vaz Cardoso Machado[a], Antônio Ávila Fernandes Júnior[a], Alberto Thalison Silveira[b], Diego Fernandes Vilas Boas[c], Elda Gonçalves dos Santos[c], Ivo Santana Caldas[c], Diogo Teixeira Carvalho[a,*]

[a]Departamento de Alimentos e Medicamentos, Faculdade de Ciências Farmacêuticas, Universidade Federal de Alfenas, Alfenas, MG, Brazil
[b]Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade Federal de Alfenas, Alfenas, MG, Brazil
[c]Departamento Patologia e Parasitologia, Instituto de Ciências Biomédicas, Universidade Federal de Alfenas, Alfenas, MG, Brazil

**Corresponding author: Diogo Teixeira Carvalho. Faculdade de Ciências Farmacêuticas, Universidade Federal de Alfenas, Rua Gabriel Monteiro da Silva, 700, sala D-208e, Alfenas, MG, 37130-001, Brazil. E-mail: diogo.carvalho@unifal-mg.edu.br. Phone number: +55 35 3701-9213*

Abstract

Natural and synthetic coumarins have been described as prototypes of new drug candidates against Chagas' disease. During a typical screening with new compounds, we observed the potential of a new synthetic nitrobenzoylcoumarin (**1**) as trypanocidal against *T. cruzi*

epimastigotas. Then we decided to prepare and evaluate a set of analogues from **1** to check the major structural requirements for trypanocidal activity. The structural variations were conducted in six different sites on the original compound and the best derivative (**3**) presented activity (IC_{50} 28 ± 3 μM) similar to that of benznidazole (IC_{50} 25 ± 10 μM). The enhancement of trypanocidal activity was conditioned to a change in the side chain at C6 (allyl to *n*-propyl group) and the preservation of coumarin nucleus and the nitrobenzoyl group at C3. Exposure of **3** to H9C2 cells showed low toxicity (CC_{50}>200 μM) and its activity on *T. cruzi* amastigotes (IC_{50} 13 ± 0.3 μM) encouraged us to perform an evaluation of its potential when given orally to mice infected with trypomastigote forms. Derivative **3** was able to reduce parasitemia when compared to the group of untreated animals. Taken together, these results show the potential therapeutic application of the synthetic coumarins.

1. Introduction

Chagas disease is considered one of the most important parasitic diseases in South and Central America and it is estimated that in the world there are approximately 7 million people infected by this parasite, with around 7000 deaths per year [1]. Chagas disease is considered a neglected disease and currently only two drugs are recommended for treatment: nifurtimox and benznidazole [2]. In addition to the efficacy of these drugs being dependent on the stage of infection in which treatment is introduced, they induce serious side effects in patients, so considering the risk of therapeutic failure and death during the chronic phase, the development of new drugs may be important not only to increase the cure rate of patients but also to prevent the progression of the disease to severe clinical forms, even if parasitological cure is not obtained [3].

Natural product research represents an important strategy in finding new drug candidates. The pharmacological activity of these substances can be optimized by rationally oriented structural modifications or they may assist as inspiration for new compounds with activities other than the original. Coumarins are good examples of this, since besides being ubiquitous in plant species, they present a wide range of pharmacological activities, as anti-inflammatory, antioxidant, antiproliferative and antimicrobial. In addition, they can be easily accessed by organic synthetic methodologies, which avoids issues inherent to obtaining bioactive molecules by exploitation of natural resources [4, 5].

In the last years, some authors have revealed the trypanocidal potential of natural and synthetic coumarins (Scheme 1) and this activity seems to be related mostly to the inhibition of trypanothione reductase (TR) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH),

which are essential enzymes in *T. cruzi* metabolic pathways [5, 6]. For instance, the prenylated coumarin Mammea B/BA (**A**) extracted from *Calophyllum brasiliense* showed good in vitro trypanocidal activity [7, 8], while chalepin (**B**), a coumarin present in the crude extracts of Rutales species, is active as GAPDH inhibitor from *T. cruzi* [4, 9]. Umbelliferone aminoalkyl derivative (**C**) inhibits the activity of oxidosqualene cyclase, an enzyme involved in *T. cruzi* ergosterol biosynthesis [10]. Ethanolic extracts from *Polygala sabulosa* have activity against epimastigote and trypomastigote forms of *T. cruzi* and this is associated to the presence of 6-methoxy-7-prenyloxycoumarin (**D**) [11]. *Ferula narthex* exudate contains the trypanocidal coumarins fesolol (**E**) and 10'-R-karatavacinol (**F**) [12]. Some synthetic coumarins like 3-amino-4-hidroxi-6-metil-2H-cromen-2-ona (**G**) showed dual activity as trypanocidal and antioxidant agents [13]. Other coumarins were synthetized as hybrids of chalcones (**H**) or quinones (**I**) and interesting trypanocidal or antioxidant activity was observed [14-17].

<Scheme 1>

Following our interests in the synthesis of new bioactive coumarins [18] and taking advantage of abundant natural products as eugenol, recently we synthesized a series of new unpublished coumarins structurally related to eugenol in order to find new antibacterial and antifungal compounds. They did not show relevant activity on these microorganisms, but one of these coumarins (**1**, Scheme 2) presented good trypanocidal effect (IC_{50} 66 μM) in a screening against epimastigote forms of *T. cruzi*, besides having low citotoxicity (>200 μM). This result encouraged us to accomplish structural changes in this coumarin in order to obtaining derivatives that could be more effective and to understand the current structure-activity relationships (SAR). For this purpose, it was decided to promote structural changes in **1** in order to investigate the influence of hydrophobicity and of electronic and steric effects on trypanocidal activity.

<scheme 2>

2. Methods and materials

2.1 Chemistry

All required chemicals were purchased from Sigma-Aldrich (São Paulo, Brazil) and used without further purification. Thin-layer chromatography (TLC) on silica gel TLC plates (ALUGRAM® Xtra Sil G/UV_{254}, Macherey-Nagel) was used to check purity of the obtained

compounds and to monitor the reactions progress. The spots were detected by exposure to the UV light at 254 nm. For column chromatography, column grade (0.040-0.063 mm mesh size) silica gel was employed (Sorbiline®). Melting points of the compounds were obtained on PFM-II melting-point apparatus (MS Tecnopon, Piracicaba, Brazil) and are uncorrected. IR spectra were recorded on FT-IR-Affinity-1 spectrometer with an ATR dispositive (Shimadzu®, Kyoto, Japan). NMR spectra were recorded on a Bruker AC-300 spectrometer operating at 300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR spectra (Rheinstetten, Germany). Chemical shifts are expressed as δ (ppm) relative to TMS as internal standard. The coupling constants (*J*) are given in Hertz. Multiplets are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), sex (sextet) and m (multiplet). Ultra-high performance liquid chromatography system (UHPLC) model LC–MS 8030 equipped with a triple-quadrupole mass analyzer was employed for compounds characterization (Shimadzu®, Kyoto, Japan). The MS conditions were as follows: source block, 400°C; desolvation line, 250ºC; nebulizing gas, 2.0 L min^{-1}; drying gas, 15.0 L min^{-1}; electrospray ionization mode (ESI). The acquisition of the precursors were in full scan (positive and negative ionization mode, ranged from 200 to 500 m/z). Two microliters of solutions at 5 ppm of each analyte dissolved in methanol were injected into the system. The mobile phase was MeOH:water (95:5, v/v) at a flow rate of 0.2 mL min^{-1}. ClogP values of synthesized compounds were calculated using ChemDraw Ultra 11.0. Coumarin **1** was synthesized as reported elsewhere [19].

2.1.1 General procedure for the synthesis of 5-allyl-2-hydroxy-3-methoxybenzaldehyde and 2-hydroxy-3-methoxy-5-propylbenzaldehyde [20]

Hexamine (5 eq) was solubilized in glacial acetic acid (40 mL) and this solution was stirred at 125 ºC for 10 minutes, then the specific phenol (1 eq) was added in one portion. Reaction was maintained at 125 °C for five hours under stirring. Following, 2M HCl (10 mL) was added and the reaction mixture was maintained as such for additional 30 minutes. Then, this mixture was cooled down to room temperature, washed with aqueous $NaHCO_3$ and extracted with dichloromethane. The organic phase solvent was evaporated in a rotary evaporator and the oil purified by column chromatography (hexanes:ethyl acetate, 9:1, v/v).
5-allyl-2-hydroxy-3-methoxybenzaldehyde

From 4-allyl-2-methoxyphenol. Light greenish oil; yield 57%. IR (ATR) 3200-2800 (ν O-H phenol), 3005 (ν C-H ar), 2965 (ν C-H sp^3), 2850 (ν C-H aldehyde), 1655 (ν C=O aldehyde), 1593 (ν C=C ar), 1265 (ν C-O-C). ^{1}H NMR ($CDCl_3$) 3.37 (d, $J_{H9,H10}$ 6.6 Hz; 2H), 3.90 (s; 3H),

5.07–5.13 (m; 1H), 5.87–6.01 (m; 2H), 6.94 (s; 1H), 6.97 (s; 1H), 9.87 (s; 1H), 10.96 (s; 1H). ^{13}C NMR ($CDCl_3$) 39.53, 56.41, 116.66, 118.82, 120.58, 123.83, 131.55, 136.85, 148.34, 150.19, 196.73.

2-hydroxy-3-methoxy-5-propylbenzaldehyde

From 2-methoxy-4-propylphenol. Yellowish oil; yield: 61%. IR (ATR) 3350-2750 (ν O-H phenol), 3003 (ν C-H ar), 2957 (ν C-H sp^3), 2870 (ν C-H aldehyde), 1647 (ν C=O aldehyde), 1595 (ν C=C ar), 1259 (ν C-O-C). ^1H NMR ($CDCl_3$) 0.95 (t; $J_{H11,H10}$ 7.39 Hz; 3H), 1.64 (sex; $J_{H10,H9}$ 7.53 Hz; $J_{H10,H11}$ 7.39 Hz; 2H), 2.57 (t; $J_{H9,H10}$ 7.53 Hz; 2H), 3.91 (s; 3H), 6.94 (s; 1H), 6.96 (s; 1H), 9.88 (s; 1H), 10.91 (s; 1H). ^{13}C NMR ($CDCl_3$) 13.80, 24.60, 37.47, 56.45, 118.89, 120.56, 123.72, 134.24, 148.19, 149.86, 196.83.

*2.1.2 General procedure for the synthesis of derivatives **2** and **3***

To a stirred solution of a specific salicylaldehyde (1 eq) in ethanol (20 mL) it was added ethyl 4-nitrobenzoylacetate (1 eq) and piperidine (3 drops). Then the mixture was heated under reflux at 80 °C during three hours when a white solid precipitated as product. Then, the mixture was cooled to room temperature and the solid product formed was collected by vacuum filtration, washed throughout with cold ethanol and dried in a desiccator.

8-methoxy-3-(4-nitrobenzoyl)-2H-chromen-2-one (**2**)

From *ortho*-vanillin. Light greenish solid; yield: 89%; CLogP 2.80; M.p.: 266-267 ºC. IR (ATR) 3105 (ν C-H ar), 3075 (ν C-H sp^2), 1698 (ν C=O ester), 1660 (ν C=O ketone), 1574 (ν C=C ar), 1514 cm^{-1} (ν NO_2). ^1H NMR (DMSO-d_6) 3.95 (s, 3H), 7.37 (t, $J_{H6,\ H5}$ 7.65 Hz; $J_{H6,H7}$ 7.92 Hz, 1H), 7.43-7.48 (m, 2H), 8.15 (d, $J_{H3',H2'}$ 9 Hz, 2H), 8.33 (d, $J_{H2',H3'}$ 9 Hz, 2H), 8.56 (s, 1H); ^{13}C NMR (DMSO-d_6) 11.31, 56.28, 118.81, 121.20, 123.70, 124.97, 125.45, 130.64, 141.41, 143.86, 146.48, 147.65, 150.09, 157.81, 190.95. MS (ESI) calcd for $C_{17}H_{11}NO_6$ $[M+H]^+$ 326.27 found 326.40.

8-methoxy-3-(4-nitrobenzoyl)-6-propyl-2H-chromen-2-one (**3**)

From 2-hydroxy-3-methoxy-5-propylbenzaldehyde. White solid; yield 91%; CLogP 4.32; M.p.: 143-144 ºC. IR (ATR) 3069 (ν C-H ar), 3048 (ν C-H sp^2), 1705 (ν C=O ester), 1682 (ν C=O ketone), 1582 (ν C=C ar), 1513 cm^{-1} (ν NO_2). ^1H NMR (DMSO-d_6) 0.98 (t, $J_{H15,H14}$ 7.37 Hz, 3H), 1.69 (sex, $J_{H14,H13}$ 7.5 Hz; $J_{H14,H15}$ 7.37 Hz, 2H), 2.67 (t, $J_{H13,H14}$ 7.5 Hz, 2H), 3.99 (s, 3H), 7.01 (d, $J_{H7,H5}$ 1.71 Hz, 1H), 7.05 (d, $J_{H5,H7}$ 1.71 Hz, 1H), 7.95-7.99 (m , 2H), 8.23 (s, 1H),

8.28-8.33 (m, 2H); ^{13}C NMR (DMSO-d_6) 13.67, 24.43, 37.68, 56.40, 116.86, 118.40, 119.91, 123.68, 125.58, 130.18, 140.20, 141.52, 143.24, 147.05, 148.12, 150.39, 190.74. MS (ESI) calcd for $C_{20}H_{17}NO_6$ $[M+H]^+$ 368.35 found 368.00.

*2.1.3 Synthesis of 3-(4-aminobenzoyl)-8-methoxy-6-propyl-2H-chromen-2-one (**4**)*

Derivative **3** (1 eq) and stannous chloride dihydrate (5 eq) were added to ethanol (20 mL) and the mixture was stirred under heating to 85 ºC under reflux. After two hours, TLC analysis (hexanes:ethyl acetate, 7:3, v/v) showed the reaction completion and the mixture was cooled to room temperature. Aqueous $NaHCO_3$ was then added to raise pH to 8 and the mixture was extracted with ethyl acetate. The resulting organic phase was washed with brine, dried with anhydrous sodium sulfate and the solvent evaporated in a rotary evaporator.

Yellow solid; yield 91%; CLogP 3.6; M.p.: 215-216 ºC. IR (ATR) 3491 (ν NH_2), 3046 (ν C-H ar), 2952 (ν C-H sp^2), 1702 (ν C=O ester), 1645 (ν C=O ketone), 1580 cm^{-1} (ν C=C ar). ^{1}H NMR (DMSO-d_6) 0.96 (t, $J_{H15,\ H14}$ 7.33 Hz, 3H), 1.67 (sex, $J_{H14,H13}$ 7.57 Hz; $J_{H14,\ H15}$ 7.33 Hz, 2H), 2.64 (t, $J_{H13,H14}$ 7.57 Hz, 2H), 3.97 (s, 3H), 4.23 (s, 2H), 6.61-6.64 (m, 2H), 6.92 (d, $J_{H7,H5}$1.72 Hz, 1H), 6.97 (d, $J_{H5,H7}$ 1.72 Hz, 1H), 7.71-7.76 (m, 2H), 7.89 (s, 1H); ^{13}C NMR (DMSO-d_6) 13.69, 24.48, 37.71, 56.34, 113.75, 115.52, 118.70, 119.28, 126.33, 128.25, 132.56, 139.55, 144.21, 146.92, 152.04, 158.41, 189.55. MS (ESI) calcd for $C_{20}H_{19}NO_4$ $[M+H]^+$ 338.37 found 338.05.

*2.1.4 Synthesis of 4-((4-(8-methoxy-2-oxo-6-propyl-2H-chromene-3-carbonyl)phenyl)amino)-4-oxobutanoic acid (**5**)*

To a stirring solution of succinic anhydride (5 eq) in pyridine (5 mL) it was added the amino derivative **4** (1 eq) and then the reaction mixture was heated at 90 ºC for 24 hours. When all amine was consumed (TLC with hexanes: ethyl acetate: methanol, 6:3.75:0.25, v/v/v), the reaction mixture was cooled to room temperature and the pH brought to 2 by addition of 1M HCl. The formed solid was collected by vacuum filtration and was purified by trituration with ethyl ether.

Brown solid; yield 42%; CLogP 3.47 ± 0,75; M.p 202-203 ºC. IR (ATR) 3278 (ν N-H amide), 3058 (ν C-H sp^2), 3000-2800 (ν O-H carboxylic acid), 2962 (ν C-H ar), 1719 (ν C=O carboxylic acid), 1710 (ν C=O ester), 1677 (ν C=O amide), 1651 (ν C=O ketone), 1584 com^{-1}

(ν C=C ar). ^{1}H NMR (DMSO-d_6) 0.86 (t, $J_{H15,H14}$ 7.33 Hz, 3H), 1.58 (sex, $J_{H14,H13}$ 7.53 Hz; $J_{H14,H15}$ 7.33 Hz, 2H), 2.44-2.59 (m, 6H), 3.88 (s, 3H), 7.12 (d, $J_{H7,H5}$ 1.56 Hz, 1H), 7.20 (d, $J_{H5,H7}$ 1.62 Hz, 1H), 7.67 (d, $J_{H3',H2'}$ 8.8 Hz, 2H), 7.83 (d, $J_{H2',H3'}$ 8.8 Hz, 2H), 8.20 (s, 1H), 10.33 (s, 1H). ^{13}C NMR (DMSO-d_6) 13.61, 24.01, 28.65, 31.26, 36.88, 56.24, 116.07, 118.23, 118.49, 119.62, 126.93, 130.40, 131.14, 139.17, 141.73, 144.41, 144.74, 146.25, 158.00, 170.99, 173.79, 190.28. MS (ESI) calcd for $C_{24}H_{23}NO_7$ $[M-H]^+$ 436.44 found 436.25.

*2.1.5 General procedure for the synthesis of derivatives **6**, **7** and **8***

A specific β-ketoester or malonic acid (1 eq), 2-hydroxy-3-methoxy-5-propylbenzaldehyde (1 eq) and L-proline (0.1 eq) were mixture and heated to 90 °C in a glycerol bath when all the solids melt. The heating was continued for an hour when TLC indicated the end of the reaction (hexane:ethyl acetate; 8:2, v/v). The remaining slurry solid was purified by trituration with diethyl ether until a fine powder was formed, which was isolated by simple filtration.

3-benzoyl-8-methoxy-6-propyl-2H-chromen-2-one (**6**)

From benzoyl acetoacetate. Light yellow solid; yield 65%; CLogP 4.36; M.p. 140-141 ºC. IR (ATR) 3051 (ν C-H ar), 2953 (ν C-H sp^2), 1716 (ν C=O ester), 1676 (ν C=O ketone), 1579 cm^{-1} (ν C=C ar); ^{1}H NMR (DMSO-d_6) 0.97 (t, $J_{H15,\ H14}$ 7.35 Hz, 3H), 1.68 (sex, $J_{H14,\ H13}$ 7.58 Hz, $J_{H14,\ H15}$ 7.35 Hz, 2H), 2.65 (t, $J_{H13,\ H14}$ 7.58 Hz, 2H), 3.98 (s, 3H), 6.95 (s, 1H), 7.00 (s, 1H), 7.44-7.49 (m, 2H), 7.60 (tt, $J_{H4',H3'}$ 7.39 Hz, $J_{H4',\ H2'}$ 2.65 Hz, 1H), 7.85-7.89 (m, 2H), 8.02 (s, 1H). ^{13}C NMR (DMSO-d_6) 13.68, 24.46, 37.70, 56.36, 116.06, 118.50, 119.54, 127.16, 128.56, 129.62, 133.74, 136.31, 139.76, 142.85, 145.82, 146.98, 191.92. MS (ESI) calcd for $C_{20}H_{18}O_4$ $[M+H]^+$ 323.35 found 323.10.

3-acetyl-8-methoxy-6-propyl-2H-chromen-2-one (**7**)

From ethyl acetoacetate. Dark yellow solid; yield 61%; CLogP 2.52; M.p. 109-110 ºC. IR (ATR) 3069 (ν C-H sp^2), 2998 (ν C-H ar), 1717 (ν C=O ester), 1676 (ν C=O ketone), 1570 cm^{-1} (ν C=C ar). ^{1}H NMR ($CDCl_3$) 0.96 (t, $J_{H15,\ H14}$ 7.38 Hz, 3H), 1.68 (sex, $J_{H14,\ H13}$ 7.56 Hz, $J_{H14,\ H15}$ 7.38 Hz, 2H), 2.64 (t, $J_{H13,\ H14}$ 7.56 Hz, 2H), 3.97 (s, 3H), 7.00 (s, 1H), 7.26 (s, 1H), 8.44 (s, 1H). ^{13}C NMR ($CDCl_3$) 13.79, 24.55, 30.76, 37.79, 56.49, 116.90, 118.70, 120.57, 124.67, 139.90, 143.54, 146.92, 148.02, 159.11, 195.95. MS (ESI) calcd for $C_{15}H_{16}O_4$ $[M+H]^+$ 261.29 found 261.10.

8-methoxy-2-oxo-6-propyl-2H-chromene-3-carboxylic acid (**8**)

From malonic acid. Yellow solid; yield 67%; CLogP 2.76; M.p. 158-159 ºC. IR (ATR) 3045 (ν C-H sp^2), 3003 (ν C-H ar), 3000-2700 (ν O-H carboxylic acid), 1742 (ν C=O ester), 1671 (ν C=O carboxylic acid), 1582 cm^{-1} (ν C=C ar). ^{1}H NMR ($CDCl_3$) 0.95 (t, $J_{H15,\ H14}$ 7.35 Hz, 3H), 1.67 (sex, $J_{H14,\ H13}$ 7.57 Hz, $J_{H14,\ H15}$ 7.35 Hz, 2H), 2.66 (t, $J_{H13,\ H14}$ 7.57 Hz, 2H), 3.98 (s, 3H), 7.05-7.06 (m, 1H), 7.09-7.10 (m, 1H), 8.82 (s, 1H). ^{13}C NMR ($CDCl_3$) 13.73, 24.44, 37.73, 56.54, 114.97, 117.98, 118.90, 120.44, 141.41, 142.60, 147.16, 151.81, 162.86, 163.85. MS (ESI) calcd for $C_{14}H_{14}O_5$ $[M-H]^+$ 261.26 found 260.95.

*2.1.6 Synthesis of 8-methoxy-3-(4-nitrobenzoyl)-6-propylchroman-2-one (**9**)*

The nitro derivative **3** (1 eq), sodium cianoborohydride (1.1 eq) and bromocresol green (1 mg) were added in methanol (10 mL). The mixture was kept at room temperature under stirring while 1M HCl was added dropwise until the solution color changed to yellow. The reaction mixture was maintained as such until TLC ((hexanes:ethyl acetate; 8:2, v/v) showed the reaction ended after 3 hours. Then, the reaction mixture was diluted with water, cooled and extracted with dichloromethane. The organic phase was dried with anhydrous sodium sulfate and the solvent evaporated in a rotary evaporator.

White solid; yield 61%; CLogP 4,00; M.p.136-137 ºC. IR (ATR) 3106 (ν C-H ar), 1738 (ν C=O ester), 1692 (ν C=O ketone), 1601 (ν C=C ar), 1521 cm^{-1} (ν NO_2). ^{1}H (DMSO-d_6) 0.94 (t, $J_{H15,\ H14}$ 7.31 Hz, 3H), 1.63 (sex, $J_{H14,\ H13}$ 7.6 Hz; $J_{H14,\ H15}$ 7.31 Hz, 2H), 2.55 (t, $J_{H13,\ H14}$ 7.6 Hz, 2H), 3.07 (dd, $J_{H4^{**}.H4^{*}}$ 15,9 Hz, $J_{H4^{**}.H3}$ 5,9 Hz; 1H), 3.56 (dd, $J_{H4^{*}.H4^{**}}$ 15,9 Hz, $J_{H4^{*}.H3}$ 5,2 Hz; 1H), 3.89 (s, 3H), 4.61 (dd; $J_{H3.H4^{*}}$ 5,2 Hz, $J_{H3.H4^{**}}$ 5,9 Hz; 1H), 6.61 (s, 1H), 6.70 (s, 1H), 8.12 (d, $J_{H2',H3'}$ 8.56 Hz, 2H), 8.32 (d, $J_{H3',H2'}$ 8.56 Hz, 2H). ^{13}C NMR (DMSO-d_6) 19.90, 24.71, 26.69, 37.97, 47.52, 56.21, 111.80, 119.38, 122.07, 124.10, 130.02, 140.25, 140.55, 147.41, 165.32, 174.18, 192.75. MS (ESI) calcd for $C_{20}H_{19}NO_6$ $[M-H]^+$ 368.37 found 368.10.

*2.1.7 Synthesis of 8-hydroxy-3-(4-nitrobenzoyl)-6-propyl-2H-chromen-2-one (**10**)*

Anhydrous aluminum chloride (1.1 eq) was added under stirring to a cold solution of the nitro derivative **3** (1 eq) in anhydrous dichloromethane. The reaction mixture was allowed to warm up to room temperature and was maintained as such for 1.5 hours, until TLC showed the total consumption of the starting material (hexanes:ethyl acetate, 7:3, v/v). The reaction was quenched by slow addition of aqueous sodium bicarbonate and the product was

extracted with ethyl acetate, followed by drying with anhydrous sodium sulfate and elimination of the solvent in a rotary evaporator.

Greenish solid; yield 79%; CLogP 4.15; M.p. 213-214 ºC. IR (ATR) 3367 (ν OH phenol), 3113 (ν C-H sp^2), 3083 (ν C-H ar), 1725 (ν C=O ester), 1654 (ν C=O ketone), 1576 (ν C=C ar), 1521 cm^{-1} (ν NO_2). ^{1}H NMR ($CDCl_3$) 0.86 (t, $J_{H15, H14}$ 7.28 Hz, 3H), 1.56 (sex, $J_{H14, H13}$ 7.45 Hz, $J_{H14, H15}$ 7.28 Hz, 2H), 2.52 (t, $J_{H13, H14}$ 7.45 Hz, 2H), 7.04 (s, 1H), 7.09 (s, 1H), 8.10 (d, $J_{H2', H3'}$ 8.71 Hz, 2H), 8.29 (d, $J_{H3', H2'}$ 8.71 Hz, 2H), 8.42 (s, 1H), 10.30 (s, 1H). ^{13}C NMR ($CDCl_3$) 16.50, 23.89, 36.49, 118.83, 119.29, 120.72, 123.68, 125.06, 130.57, 139.13, 141.56, 144.35, 147.91, 150.01, 158.12, 191.12. MS (ESI) calcd for $C_{20}H_{19}NO_6$ $[M-H]^+$ 368.37 found 368.10. MS (ESI) calcd for $C_{19}H_{15}NO_6$ $[M-H]^+$ 352.33 found 352.10.

*2.1.8 Synthesis of 3-(hydroxyl-(4-nitrophenyl)methyl)-8-methoxy-6-propyl-2H-chromen-2-one (**11**)*

Sodium borohydride (1.1 eq) was solubilized in 0.01M NaOH (5 mL) and this was added gradually to a stirred solution of the nitro derivative **3** (1 eq) in metanol (10 mL) at room temperature. After 3 hours, when the reaction was over (TLC, hexanes:ethyl acetate, 7:3, v/v), HCl 1M (10 mL) was added and the solution allowed to stir for another 30 minutes. Then, the reaction mixture was neutralized with aqueous sodium bicarbonate and extracted with ethyl acetate. After drying the organic phase with anhydrous sodium sulfate and elimination the solvent in rotary evaporator, the final slurry was triturated with diethyl ether to afford the product.

Light brown solid; yield 71%; CLogP 4.83; M.p.180-181 ºC. IR (ATR) 3378 (ν OH alcohol), 3113 (ν C-H sp^2), 3067 (ν C-H ar), 1683 (ν C=O ester), 1588 (ν C=C ar), 1522 cm^{-1} (ν NO_2). ^{1}H NMR ($CDCl_3$) 0.89 (t, $J_{H15, H14}$ 7.3 Hz, 3H), 1.62 (sex, $J_{H14, H13}$ 7.53 Hz, $J_{H14, H15}$ 7.3 Hz, 2H), 2.59 (t, $J_{H13, H14}$ 7.53 Hz, 2H), 3.87 (s, 3H), 5.77 (d, $J_{H16, OH}$ 4.24 Hz, 1H), 6.41 (d, $J_{OH, H16}$ 4.24 Hz, 1H), 7.13 (s, 1H), 7.16 (s, 1H), 7,68 (d, $J_{H2', H3'}$ 8.75 Hz, 2H), 8.13 (s, 1H), 8.18 (d, $J_{H3', H2'}$ 8.75 Hz, 2H). ^{13}C NMR ($CDCl_3$) 13.58, 23.97, 36.90, 56.07, 68.79, 114.46, 118.90, 119.08, 123.36, 128.13, 130.71, 138.91, 140.34, 146.11, 146.76, 150.26, 159.25. MS (ESI) calcd for $C_{20}H_{19}NO_6$ $[M-H]^+$ 368.37 found 368.00.

2.2 Biological evaluation

2.2.1 Trypanosoma cruzi strain and culture procedure

The *T. cruzi* Y strain (DTU II), partially sensitive to benznidazol chemotherapy and characterized by Filardi and Brener was used [21]. The epimastigote forms were maintained in culture at 28 °C in LIT (Liver Infusion Triptose) medium, supplemented with 10% fetal bovine serum (FBS). The trypomastigote forms of strain Y were maintained in liquid nitrogen and periodically transferred to mice of the Swiss line, during successive passages. The strain maintenance procedure was carried out at the Animal Facility of the Pathology and Parasitology Department of the Federal University of Alfenas (UNIFAL-MG) to avoid changes in the original characteristics of susceptibility and virulence.

2.2.2 In vitro anti-Trypanosoma cruzi assay

Stock solutions of coumarins and benznidazole (10 mg/mL) were prepared in dimethyl sulfoxide (DMSO). For analysis of the effects against epimastigotes forms, the maximum concentration was 200 µg/mL for each compound and benznidazole, and a seven-point profile with 2-fold serial dilutions was used in triplicate. Then, epimastigotes were added to the plate (1.5 x 10^6/mL) in a 72-h assay. The final DMSO concentration had no deleterious effect on parasite growth. After incubation in a BOD oven at 28 °C for 72 hours, resazurin (1mM) was added to the plates. After incubation for 12 hours, the results were read at 570 nm and 600 nm. The percentage of inhibition of proliferation induced by the compounds was calculated using the formula: % inhibition = 100 – [A_{570}-(A_{600}xR0) $_{Treated}$ / A_{570}-(A_{600}xR0) $_{Control+}$] x100, where A_{570}= Absorbance a 570nm, A_{600}= Absorbance a 600nm, where Control + represents the well containing epimastigotes, medium and resazurin, in the absence of the compound. R0 is the correction factor, calculated from the absorbance values of the negative control (C-), that is, only culture medium and resazurin in the absence of parasites [Ro = (A_{570} /A_{600})$_{C-}$]. The IC_{50} values were calculated from the percent inhibition of compounds using Graph Pad Prism 5 software.

For analysis of the effects against intracellular parasites, the concentrations used were 120µM, 60µM, 30µM, 15µM, 7.5µM, 3.75µM and 1.87µM for 4-nitrobenzoyl coumarin or benznidazole, in a 72 hours assay. Cells of H9c2 lineage (1x10^4 cells; American Type Culture Collection, ATCC: CRL 1446) from neonatal rat cardiomyoblasts were cultured in DMEM medium (supplemented with 10% FBS, 1% glutamine 2nM and 0.2% gentamicin 200µg / ml). After 24 h, the cells were infected with trypomastigotes (obtained from H9c2

cells) at a ratio of 20 parasites to 1 H9c2 cell. After 24 h of incubation, the cultures were exposed to the compounds for 3 days. All tissue culture plates were maintained at 37°C in a 5%CO_2-air mixture. After 72 h, the cultures were fixed with methanol, stained with Giemsa stain, and examined microscopically to determine the percentages of infected cells in treated and untreated cultures. At least 200 cells were counted for determination of percentage of infection and the percentage of growth inhibition were obtained by dividing the number of infected cells in treated cultures by the mean value for infected control cultures. Subsequently this result was multiplied by 100 [22]. IC_{50} values were calculated using CalcuSyn software (Biosoft, United Kingdom) and Graph Pad Prism 5.

2.2.3 In vitro cell toxicity assay

Cells of H9c2 Lineage (1 X 10^3) were cultured in DMEM medium (supplemented with 10% FBS, 1% glutamine 2nM and 0.2% gentamicin 200µg / ml). 1000 H9c2 cells were seeded and incubated at 37°C, 5% CO_2 for 24 hours. The culture medium was replaced and for analysis of the effects toxic, the maximum concentration was 200 µg/mL for each compound and benznidazole, and a seven-point profile with 2-fold serial dilutions was used in triplicate for 72 hours. After incubation, resazurin (1mM) was added and the reading was carried out at 570nm and 600nm after 12 hours hours [23]. The percentage of inhibition of proliferation induced by the compounds was calculated using the same formula used in the assays against epimastigote forms. From the percent inhibition of the compounds CC_{50} (50% cytotoxic concentration) was calculated using the Graph Pad Prism 5.

2.2.4 In vivo activity

Female Swiss mice with about 30 days of age (20-25g), from the UNIFAL-MG, and maintained in a temperature-controlled room with access to water and food *ad libitum* were inoculated intraperitoneally with 5x10^3 blood trypomastigotes of the *T. cruzi* Y strain. This strain is partially resistant to benznidazole and induces high levels of parasitemia accompanied by 100% mortality at around 15 days of infection when in the absence of treatment. After four days, tail blood was examined for the presence of parasites. Only when *T. cruzi* was detected microscopically, were the mice submitted to a specific treatment. The treatment was administered orally at the dosage of 50mg/kg. The compounds (derivative **3** and benznidazole) were each suspended in distilled water using 5% de Cremophor (Sigma) and each animal received 0.2 mL of compound suspension daily by gavage for 6 consecutive days. Parasitaemia and mortality were checked every day until ten days after infection.

2.2.5 Ethics

All experimental protocols were conducted in accordance with the guidelines issued by the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethics Committee in Animal Research at UNIFAL-MG (number 59/2017).

3. Results and discussion

3.1 Chemistry

The synthetic plan to obtain the intended coumarins is depicted in Scheme 3. Coumarins **1**-**3** and **6**-**8** were obtained by Knoevenagel reactions between specific aromatic aldehydes and β-ketoesters (**1**-**3**, **6** and **7**) or malonic acid (**8**) employing the conditions described by Karade et al (2007) or Vazquez-Rodriguez (2013) [24, 24]. They were all obtained as pure and crystalline solids with good yields. The analysis of their IR spectra showed bands between 1742-1645 cm^{-1} assigned to the carbonyl groups (lactone, ketone or carboxylic acid). In addition, all their ^{1}H NMR spectra showed the signal for H-4 of the coumarin nucleus in the range 8.82-7.89 ppm. The confirmation of coumarins formation was also due to the characteristic signals of two carbonyl carbons, found above 150 ppm, in their ^{13}C NMR spectra.

To obtain de amino derivative **4** it was employed the methodology proposed by Bellamy and co-workers which uses stannous chloride in ethanol [26]. In this way, coumarin **4** was smoothly obtained in high yields. The IR stretching bands at 3491 e 3373 cm^{-1} and an expressive shielding effect on aromatic hydrogens noted in the NMR spectrum confirmed the presence of amino group. The succinamic derivative **5** was synthesized by reacting the amino coumarin **4** with succinic anhydride in refluxing pyridine. In its IR spectrum it was possible to note the four expected carbonyl bands due to lactone, ketone, amide and carboxylic acid groups. The insertion of the succinoyl chain was also confirmed by the presence of a multiplete in the range 2.59-2.44 ppm in the ^{1}H NMR spectra and also by the two new methylene carbon signals in the ^{13}C NMR spectrum.

<Scheme 3>

Next, sodium cianoborohydride was chosen to accomplish the reduction of the coumarin alkene group to generate derivative **9**, following what was described by Hutchins and coworkers [27]. Isolated alkenes are inert to reduction by sodium cianoborohydride, though

highly polarized double bonds, as in this case, may be reduced in acidic medium without affecting the ketone group. One can note the diastereotopic hydrogens, originated from the reduction, as double doublets in the ^{1}H NMR spectrum. On the other hand, a new methylene carbon signal was observed at 26.6 ppm in the ^{13}C spectrum. Derivative **10** was obtained by an *O*-demethylation reaction with coumarin **3** carried out by aluminum chloride in dry dichloromethane, as an adaptation of a well-known methodology [28]. The loss of methyl group could be perceived by the absence of typical methoxyl signals in NMR spectra. Finally, coumarin **11** was achieved by a classical ketone reduction made by sodium borohydride in alkaline media [29]. In its IR spectra one could see only a carbonyl stretching band whereas the ketone signal was also absent in ^{13}C NMR spectrum.

3.2 Biological evaluation

Structural analogues of coumarin **1** were devised to investigate structure-activity relationships. Firstly, we investigated the trypanocidal effect of analogues **1**-**11** against *T. cruzi* epimastigotes and the cytotoxicity of the most interesting derivatives on cardiomyocytes (Table 1).

<table 1>

It is possible to suggest that the presence of an allylic (**1**) or propylic (**3**) side chain on the coumarin nucleus positively affects the trypanocidal activity and that the saturated chain leads to a more active derivative. This result allows inferring that hydrophobic interactions between a flexible side chain of the ligand and the molecular target should be important for the activity, due to the drastic drop in activity observed with the unsubstituted derivative **2**. Since derivative **3** showed a better selectivity index than its allylic analogue **1**, it was chosen for the other intended modifications. Therefore, the next changes aimed at evaluating the pharmacophoric importance of the substituent group at the C-4' position on the benzoyl aromatic ring, besides the importance of this ring itself.

The results obtained with compounds **4** (amino derivative), **5** (succinamic derivative) and **6** (unsubstituted benzoyl derivative) showed that the nitro group, among the evaluated options, is the better substituent in the ring. This may be related to the importance of the aromatic nitro group in various antiparasitic groups (*e.g.*, nitrofurazone, benznidazole, nifurtimox, megazol, oxamniquine, praziquantel) wherein it receives the classification of parasitophoric group [30].

The results of antiepimastigote activity obtained with derivative **6**, **7** and **8**, allow us to point out the pharmacophoric importance of the benzoyl group at C-3 on the coumarin nucleus, since lower values of trypanocidal activities were found when it was replaced by methyl (**7**) or carboxyl (**8**) groups. This finding corroborates the high trypanocidal activities of coumarins in the literature with bulky substituints in this region [15, 25, 30].

From these results derivative **3** was maintained as prototype for the last three planned modifications. These aimed at an additional assessment of different functional groups in the structure and even an attempt to improve the water solubility profile of the substance in question. The drastic drop in activity observed for the derivative **9** (actually a 3,4-dihydro-benzopiran-2-one derivative) suggests the importance of a coumarin nucleus intact for the trypanocidal effect. At the same time, there was a significant increase in the cytotoxicity of this coumarin against cardiomyocytes. This alkene reduction decreases the occurrence of the flat conformation that the coumarin nucleus possessed and its increased flexibility may have negatively affected the interaction with the binding site.

Coumarin **10** (*O*-demethylated derivative) is among the most active derivatives in this work. In contrast, the exposure of the phenolic hydroxyl promoted an increase in cytotoxicity, positioning it along with derivative **9** as derivatives in which the selectivity index is totally inadequate. Thus, it was noted that the intact methoxyl group is important for the desired profile of activity with this series.

Finally, the reduction of the ketone group (derivative **11**) led to a slight decline in trypanocidal activity and increase in cytotoxicity. However, it still gave a selectivity index greater than five. The alcoholic group formed is interesting because it may be subject to new modifications, especially those that aim to increase water solubility. It is worth mentioning that the reduction products **9** and **11** were obtained as racemic mixtures, so the trypanocidal potential of isolated enantiomers may be quite different and these studies are currently in course in our group.

A comparison of the findings on anti-epimastigote and cytotoxicity activities permitted the selection of coumarin **3** as the best derivative to conduct in vitro assays against amastigote forms of *T. cruzi*. Although assays using epimastigotes are widely used for screening trypanocidal substances [23,32,33,34] some studies show divergences between the observed activity when using the three evolutionary stages of *T. cruzi* [35]. Despite the fact that correlation has been reported between compound activity against epimastigote form and *in vivo* activity using mice as experimental models [36], in this work, the evaluation of the cellular

amastigote model was carried out before performing the *in vivo* evaluation. The results obtained in this test are shown in Figure 1.

<figure 1>

There was a significant decrease in the number of cells infected by amastigote forms in both treatments, with activity observed with benznidazole treatment (IC_{50} = 3μM) similar to that observed with treatment with the derivative 3 (IC_{50} = 13μM). The decrease in the amount of infected cells can be observed as the concentration of derivative **3** and benznidazole is increased, in addition to the lower number of infected cells, it can be observed lower number of amastigotes per cell. This shows a strong effect of decreasing infectivity and multiplication of the parasite.

Thus, as derivative **3** was shown to be effective against amastigote forms and no cytotoxic effects were observed in the host cells, a preliminary in vivo evaluation of the potential efficacy of that compound in reducing parasitaemia was conducted in the sequence. This efficacy was determined using the acute murine infection model with *T. cruzi* Y strain at oral doses of 50 mg/kg for six consecutive days. Coumarin **3** was administered to the mice via suspension, since even with the use of 5% Cremophor there was no complete dissolution of the substance. The parasitaemia was reduced in 100% of the animals treated with derivative **3** or benznidazole. A reduction in parasitaemia peak and in the area under the curve in the treated group with derivative **3** can be clearly observed (Figure 2). Although the in vitro assay demonstrated efficacy of **3**, animals receiving this compound had a peak of parasitaemia with an order less than that of benznidazole. However, it is stressed that the decrease of the area under the curve was of the same order for both, although greater for benznidazole. It is worth mentioning that at the end of the tests all treated animals, both with the derivative **3** and benznidazole, presented good appearance and good health, indicating that the treatment with the substances possibly did not cause any type of apparent, physiological or external damage. The animals had normal habits and there was no death.

It should be assumed that in vitro results against epimastigote forms do not assure activity of a substance against amastigote and trypomastigote forms, due to the morphological and physiological differences among the evolutionary forms of the parasite [21]. However, the biological evaluations carried out investigated the activity of the substance against each of these forms. The parasitaemia reduction assay is accomplished during the acute phase of infection, which is characterized by the high concentration of trypomastigote forms in the

bloodstream. Derivative **3** showed a similar trypanocidal profile against the three evolutionary forms of *T. cruzi.*

<figure 2>

4. Conclusion

A significant increase in activity against *T. cruzi* epimastigote form was observed when the allyl group on the C6 carbon of nitrobenzoylcoumarin (**1**) was changed to an *n*-propyl chain, making it as active as benznidazole. This improvement in the activity was accompanied by low toxicity, suitable activity against the amastigote forms and the ability in lowering parasitaemia of mice infected by the Y strain of *T. cruzi*. Together, these results demonstrate the trypanocidal potential of coumarin **3**, which is now under studies in our group to enhance its in vivo activity and to discover its mode of action.

Acknowledgements

This work was supported by grants from Fapemig, Brazil (APQ-01268-16).

Conflict of interest

The authors declare no conflict of interest.

References

[1] WHO, Media Centre. 2017, Chagas disease (American trypanosomiasis) fact sheet.
[2] J.C.P. Dias, Ann. Int. Med. 2006, 144, 772.
[3] J.A. Urbina, Acta Trop. 2010, 115, 55.
[4] J. Alvim Jr., R.L.A. Dias, M.S. Castilho, et al. J. Braz. Chem. Soc. 2005, 16, 763.
[5] J.C. Gomez-Verjan, E. Estrella-Parra, E.R. Vazquez-Martinez, et al. Food Chem. Toxicol. 2016, 91, 117.
[6] P.C. Vieira, J. Mafezoli, M.T. Pupo, et al. Pure Appl. Chem. 2001, 73, 617.
[7] E. Scio, A. Ribeiro, T.M.A. Alves, et al. J. Nat. Prod. 2003, 66, 634.
[8] R. Reyes-Chilpa, E. Estrada-Muñiz, E. Vega-Avila, et al. Mem. Inst. Oswaldo Cruz. 2008, 103, 431.
[9] F. Pavão, M.S. Castilho, M.T. Pupo, et al. FEBS Letters. 2002, 520, 13.
[10] S. Oliaro-Bosso, F. Viola, S. Taramino, et al. ChemMedChem. 2007, 2, 226.
[11] M.G. Pizzolatti, B.G. Mendes, A. Cunha Jr, et al. Braz J. Pharmacogn. 2008, 18, 177.
[12] A. Amin, E. Tuenter, P. Cos, et al. Molecules. 2016, 21, 1287.

[13] R.F. Guíñez, M.J. Matos, S. Vazquez-Rodriguez, et al. Future Med. Chem. 2013, 5, 1911.
[14] F. Belluti, E. Uliassi, G. Veronesi, et al. ChemMedChem. 2014, 9, 371.
[15] S. Vazquez-Rodriguez, R.F. Guíñez, M.J. Matos, et al. Med. chem. 2015, 5, 173.
[16] E. Uliassi, G. Fiorani, R.L. Krauth-Siegel, et al. Eur. J. Med. Chem. 2017, 141, 138.
[17] F.Pérez-Cruz, S. Vazquez-Rodriguez, M. J.Matos, A. Herrera-Morales et al. J. Med. Chem. 2013, 56 (15), 6136
[18] I. A. Rosa, L. de Almeida, K.F. Alves, et al. Med Chem Res. 2016, 26, 131.
[19] V. La Pietra, L. Marinelli, S. Cosconati. Eur. J. Med. Chem. 2012, 47, 143.
[20] N. Grimblat, A.M. Sarotti, T.S. Kaufman, et al. Org. Biomol. Chem. 2016, 14, 10496.
[21] L.S. Filardi, Z. Brener, Trans. R. Soc. Trop. Med. Hyg. 1987, 81, 755.
[22] M. T. Bahia, A. F. Nascimento, A. L. Mazzeti et al. Antimicrob Agents Chemother. 2014, 58 (8), 4362.
[23] M. Rolón, C. Veja, J. A. Escario et al. Parasitol. Res. 2006, 99 (2), 103.
[24] N.N. Karade, S.V. Gampawar, S.V. Shinde, et al. Chin. J. Chem. 2007, 25, 1686.
[25] S. Vazquez-Rodriguez, R.F. Guíñez, M.J. Matos, et al. Med. Chem. Commun. 2013, 4, 993.
[26] F.D. Bellamy, K. Ou, Tetrahedron Lett. 1984, 25, 839.
[27] R.O. Hutchins, D. Rotstein, N. Natale, et al. J. Org. Chem. 1976, 41, 3328.
[28] T.M. Kosak, H.A. Conrad, A.L. Korich, et al. Eur. J. Org. Chem. 2015, 34, 7460.
[29] L. Banfi, E. Narisano, R. Riva et al. Encyclopedia of Reagents for Organic Synthesis. 2004. J. Wiley & Sons, New York.
[30] M.S. Lopes, R.C.C.S. Pietra, T.F. Borgati, et al. Eur. J. Med. Chem. 2011, 46, 5443.
[31] F. Sanchez-Sancho, N.E. Campillo, J.A. Paez, Curr. Med. Chem. 2010, 17, 423.
[32] J. A. Urbina, G. Payares, C. Sanoja et al. Int. J. Antimicrob Agents. 2003, 21 (1), 27.
[33] M. A. Chena, S. Elizondo, L. Rodríguez-Páez et al. J. Enzyme Inhib. Med. Chem. 2005, 20 (2),189.
[34] M. Moreno, D. A. D'ávila, M. N. Silva et al. Mem. Inst. Oswaldo Cruz. 2010, 105 (7), 918.
[35] R. A. Neal, J. van Bueren. Trans. R. Soc. Trop. Med. Hyg. 1988, 82 (5), 709.
[36] S. L. Croft. Parasitol. Today.1986, 2, 64.

Scheme 1: Natural and synthetic coumarins with trypanocidal activity. A) mammea B/BA type coumarin; B) chalepin; C) umbelliferone aminoalkyl derivative; D) 6-methoxy-7-prenyloxycoumarin; E) fesolol; F) 10'-R-karatavacinol; G) 3-amino-4-hydroxy-2*H*-chromen-2-one; H) (*E*)-3-(3-(2,5-dimethoxyphenyl)acryloyl)-2*H*-chromen-2-one; I) 2-((3-(2-(dimethylamino)ethoxy)-2-oxo-2*H*-chromen-7-yl)oxy)anthracene-1,4-dione.

Scheme 2: Coumarin **1** and structural modification planned for structure-activity relationship studies.

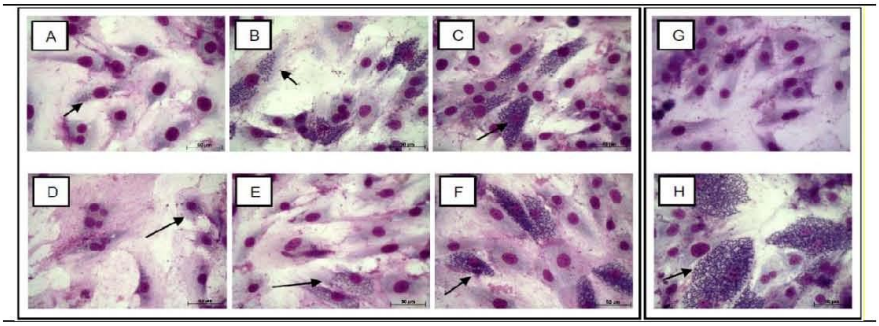
Scheme 3: Synthetic route to coumarin **1** and its analogues **2**-**11**. Reagents and conditions: (i) 1) hexamine, glacial AcOH, 125 ºC, 5h, 2) HCl 2M, 0.5h; (ii) ethyl 4-nitrobenzoyl acetate, piperidine, EtOH, 80 ºC, 3h; (iii) $SnCl_2$. H_2O, EtOH, 85 ºC, 2h; (iv) succinic anhydride, pyridine, 90 ºC, 24h; (v) benzoyl acetoacetate for derivative **6** or ethyl acetoacetate for derivative **7** or malonic acid for derivative **8**, L-proline, 90ºC, 1h; (vi) $NaBH_3CN$, HCl 1M, MeOH, r.t., 3h; (vii) $AlCl_3$, anhydrous CH_2Cl_2, r.t., 1.5h; (viii) 1) $NaBH_4$, NaOH 0.1M, MeOH, r.t., 3h, 2) HCl 1M, 0.5h.

Table 1 – Trypanocidal activity (IC_{50}, µM) against *T. cruzi* epimastigote forms, citotoxicity on cardiomyocytes H9C2 (CC_{50}, µM) and selectivity index (SI, from the ratio CC_{50}/IC_{50}) for coumarins **1**-**11** and benznidazole.

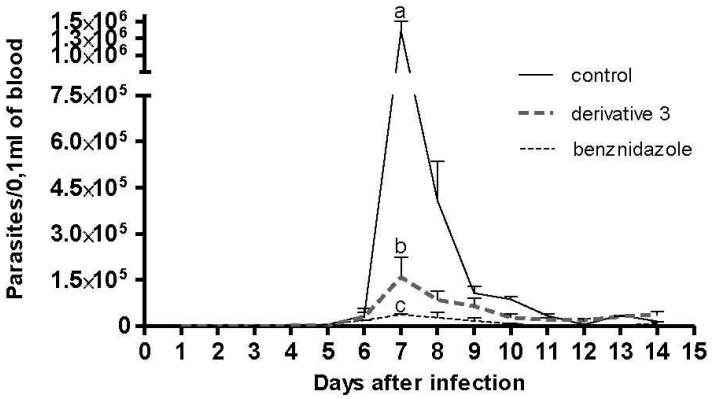
Figure 1 - Slides and activity results of coumarin **3** and benznidazole against amastigote forms of *T. cruzi*. Cells treated with coumarin **3**: 60 µM (A), 15 µM (B) and 3.75 µM (C). Cells treated with benznidazole: 60 µM (D), 15 µM (E) and 3.75 µM (F). Test controls: uninfected cells (G) and untreated infected cells (H). The arrows point to amastigotes. Bar indicates 50 µm.

Figure 2 - Influence of treatment with derivative **3** and benznidazole on parasitaemia. The lines represent the means of parasitaemia values obtained in peripheral blood samples from mice infected with 5,000 trypomastigotes of the *T. cruzi* Y strain. Control: animals infected by *T. cruzi* and untreated. AUC: area under the curve. Line different letters are significantly different ($P<0.05$).

Compound	IC_{50} ± SD	CC_{50}	SI
1	66 ± 15	>200	>3.03
2	>500	-	-
3	28 ± 3	>200	>7.14
4	>500	-	-
5	>500	-	-
6	176 ± 30	-	-
7	251 ± 43	-	-
8	318 ± 40	-	-
9	196 ± 58	108 ± 37	0.55
10	71 ± 32	58 ± 18	0.81
11	35 ± 12	185 ± 70	5.28
Benznidazole	25 ± 10	>200	>8



Compound	IC_{50} ± SD	SI
3	13 ± 3.79	>15
Benznidazole	3 ± 0.28	>66



	PEAK VALUE	AUC
Control	1.35 x 10^6	2.07 x 10^6
3	1.58 x 10^5	4.51 x 10^5
Benznidazole	3.6 x 10^4	1.20 x 10^5

`